(54) Title: MEGAKARYOCYTE PRODUCTION

(57) Abstract

A method of increasing megakaryocyte production is disclosed. Such method comprises administering to a mammal a pharmaceutically effective amount of G-CSF and a pharmaceutically effective amount of IL-3 or GM-CSF, and optionally a pharmaceutically effective amount of IL-6. Another method comprises administering GM-CSF and IL-5. Also disclosed are compositions for use in increasing megakaryocyte production. A method of increasing blood platelet production is also disclosed. Such method comprises administering to a mammal a pharmaceutically effective amount of IL-6 and optionally a pharmaceutically effective amount of IL-3, G-CSF or GM-CSF. Also disclosed are compositions for use in increasing blood platelet production.

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MEGAKARYOCYTE PRODUCTION

This invention is directed to methods of producing megakaryocytes and platelets. More
5 particularly, the subject invention is directed to treatment of disorders involving megakaryocyte deficiency and low blood platelet counts.

Background of the Invention

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Megakaryocytopoiesis is the production of megakaryocytes. Megakaryocytes are large cells of the bon: ..:row which do not normally circulate in the bloou Mature megakaryocyte cells have a greatly
15 lobulated nucleus, and mature blood platelets are released from the cytoplasm of the cells. Blood platelets (thrombocytes) which are chiefly known for their role in blood coagulation, lack a nucleus and DNA but contain active enzymes and mitochondria.
20 Studies with purified and recombinant hemopoietic factors in both human and murine systems have shown that granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin 3 (IL-3) are both capable of stimulating megakaryocyte (MK) colony
25 formation from marrow or peripheral blood derived MK progenitor cells (CFU-MK) (1-7). GM-CSF and IL-3 stimu:. : mainly small MK colonies consisting of 5-20 cells (6-8). The ability of purified or recombinant GM-CSF or IL-3 to stimulate MK colony formation (1-7),
30 and the additive effect of the two factors (3,6) is known. Granulocyte colony stimulating factor ("G-CSF") has been reported to enhance murine IL-3 dependent MK colony formation (8).

A megakaryocyte specific colony stimulating
35 activity (MK-CSA) has been reported by a number of investigators to be present in peripheral blood

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leukocyte conditioned medium (PHA-LCM) (9,10), serum and
plasma of various types of patients, in plasma of
patients with amegakaryocytic thrombocytopenia (12) or
urinary extracts from patients with aplastic anemia and
5   idiopathic thrombocytopenia purpura (11), as well as
from established cell sources (11-13). In addition,
potentiating or synergistic factors that enhance.MK
colony number, size, cellularity and maturity have been
described (8,14-16). The biochemical nature of MK-CSA
10 and these auxillary factors, however, have as yet not
been well defined.

        Tayrien and Rosenberg reported on a
megakaryocyte stimulating factor (MSF) (13) and McDonald
reported on the presence of a thrombopoietin (36), both
15 present in medium conditioned by embryonic kidney
cells. Thrombopoietin has been reported to lack direct
MK colony stimulating activity (37). That MSF is a
novel lineage specific MK-CSA with direct MK colony
stimulating activity still awaits confirmation through
20 gene cloning.

        A requirement for two or more factors
interacting to stimulate optimal in vitro MK colony
formation in both the murine and human systems have been
reported (14-16, 38). These studies implicate a
25 potentiating activity for megakaryocytopoiesis, though
the biochemical nature of the potentiating factor(s)
remains to be determined. A number of investigators
have reported on the ability of erythropoietin (EPO) to
stimulate megakaryocyte colony formation (39,40) and to
30 enhance MK cloning efficiency (41). Using highly
enriched subpopulations of hematopoietic progenitor
cells, and a serum depleted chemically defined medium,
Lu et al. were unable to show a direct effect of
EPO (6). Interleukin 4 (IL-4 or BSF-1), also reported
35 to enhance murine MK colony formation (42) was equally
ineffective.

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Summary of the Invention

The subject invention relates to a method of producing megakaryocytes. The invention comprises administering to a mammal a pharmaceutically effective amount of G-CSF and a pharmaceutically effective amount of IL-3 or GM-CSF, and optionally a pharmaceutically effective amount of IL-6. The invention also comprises administering a pharmaceutically effective amount of GM-CSF and a pharmaceutically effective amount of IL-5. The subject invention also relates to compositions comprising a pharmaceutically effective amount of G-CSF and a pharmaceutically effective amount of GM-CSF and/or IL-3 and optionally a pharmaceutically effective amount of IL-6, and compositions comprising a pharmaceutically effective amount of GM-CSF and a pharmaceutically effective amount of IL-5.

The subject invention relates to a method of producing blood platelets. The invention comprises administering to a mammal a pharmaceutically effective amount of IL-6 and optionally a pharmaceutically effective amount of IL-3, G-CSF or GM-CSF. The subject invention also relates to compositions comprising a pharmaceutically effective amount of IL-6 and a pharmaceutically effective amount of IL-3, G-CSF or GM-CSF.

Brief Description of the Drawings

Fig. 1 shows synergistic enhancement of GM-CSF dependent MK colony formation. Accessory depleted bone marrow cells were cultured at 10^4/ml in triplicate plates. GM-CSF and G-CSF were used at 1 ng/ml, and IL-5 and IL-6 at 10 ng/ml. The symbol * represents values statistically significantly different (p<0.05) from that of GM-CSF alone. The symbol + represents values

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statistically significantly different from that of
GM-CSF + G-CSF.

5   Fig. 2 shows synergistic enhancement of IL-3 dependent
MK colony formation. Accessory depleted bone marrow
cells were cultured at 10^4/ml in triplicate plates. The
symbol * represents values statistically significantly
different (p<0.05) from that of IL-3 alone. The symbol
+ represents value statistically significantly different
10   from that of IL-3 + G-CSF.

Fig. 3 shows blood platelet counts in three monkeys
receiving daily subcutaneous injections of IL-6 at
10 µg/kg for 18 days (animal #1) or 21 days (animal #2).
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Fig. 4 shows blood platelet counts in one monkey
receiving daily subcutaneous injections of IL-6 and IL-3
each at 10 µg/kg for 32 days

20   Fig. 5 shows blood platelet counts in two monkeys
receiving daily subcutaneous injections of IL-6 and
G-CSF each at 10 µg/kg for 17 days (animal #5) or 25
days (animal #6).

25   Fig. 6 shows blood platelet counts in one monkey
receiving daily subcutaneous injections of IL-6 and
GM-CSF each at 10 µg/kg for 21 days.

Plate 1 shows immuno-alkaline phosphatase negative
30   granulocyte-macrophage colonies (400x) (a & b). Low
magnification (100x) view to show 2 immuno-alkaline
phosphatase positive MK colonies among immuno-alkaline
phosphatase negative GM colonies (c). A monoclonal
murine anti-human GPIIIa antibody was used in these
35   preparations. Use of anti-FVIII gave similar results.

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Plate 2 shows small immuno-AP positive MK colony (400x)
(a). Medium sized strongly immuno-AP positive MK
colony, high magnification (1000x) (b). Large strongly
immuno-AP positive MK colony (400x) (c). A monoclonal

5   murine anti-human GPIIIa was used as the primary
antibody in these preparations. The use of anti-FVIII
yielded similar results.

Detailed Description of the Invention

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According to the subject invention, methods
for enhancing megakaryocytopoiesis are presented.
A semi-solid agarose clonal culture system
independent of human serum or plasma was used to study

15   the effect of various recombinant cytokines on human
megakaryocytopoiesis. Since various studies have
demonstrated regulatory influences of T cells, embryonic
kidney cells and NK cells on in vitro
megakaryocytopoiesis (13, 17-20), marrow cells

20   exhaustively depleted of T cells, B cells, NK cells,
macrophages, mature granulocytes and erythroid elements
(21) were used as a source of enriched megakaryocyte
precursors (CFU-MK). MK colonies were identified
in situ by immuno-alkaline phosphatase staining for the

25   MK/platelet specific markers platelet glyco-protein
GPIIIa and/or Factor VIII related antigen (FVIII)
(22-25). The examples below demonstrate a synergistic
enhancement of GM-CSF/IL-3 dependent in vitro
megakaryocytopoiesis by IL-5, G-CSF and IL-6.

30   Recombinant human granulocyte-macrophage
colony stimulating factor (GM-CSF) (Cantrell et al.,
PNAS 82, 6250 (1985)) or interleukin 3 (IL-3) (Yang
et al., Cell 47, 3 (1986)) both stimulated the formation
of small MK colonies of between 3-20 cells in a dose

35   dependent manner. Plateau levels of MK colonies were
attained at concentrations of 0.1-1 ng/ml GM-CSF or

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approximately 1-10 ng/ml IL-3. These results are in
keeping with those reported by others(5-7). The use of
accessory depleted marrow cells and heat inactivated
fetal bovine serum instead of human plasma minimized the
5 effect of endogenously produced cytokines and accessory
factors might be present in human plasma. Under such
culture conditions, GM-CSF or IL-3 induced colonies were
predominantly of small size (3-10 cells).

        Recombinant human IL-5, (Yokota et al.
10 PNAS 84, 7388 (1987)) and granulocyte colony stimulating
factor (G-CSF) (Souza et al., Science 232, 61 (1986))
did not possess MK colony stimulating activity.
However, the addition of either IL-5 or G-CSF to GM-CSF
containing cultures resulted in an increase in total
15 colony numbers as well as the appearance of larger sized
colonies of up to 50 cells. G-CSF (at 0.01-1 ng/ml),
which by itself showed no MK colony stimulating
activity, was found to significantly enhance MK colony
formation induced by GM-CSF or IL-3, resulting in both
20 increases in total colony number and cellularity.
Recombinant IL-5 which, like G-CSF had no direct MK
colony stimulating activity also showed an enhancing
effect on GM-CSF dependent megakaryocytopoiesis. IL-5
did not synergize with G-CSF in augmenting GM-CSF
25 induced MK colony formation. The effect of IL-5 on IL-3
induced MK colony formation is not presented since the
combination of IL-3 and IL-5 results mainly in
enhancement of eosinophil differentiation and colony
formation (35).
30   While IL-6 (Hirano et al., Nature 324,
73(1986)) by itself failed to enhance GM-CSF dependent
MK colony formation, it nevertheless was able to further
augment the G-CSF enhancement of GM-CSF dependent MK
colony formation. This enhancement was made by the
35 simultaneous addition of IL-6 at 1-10 ng/ml. Moreover,
the colonies stimulated by a combination of GM-CSF,

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G-CSF and IL-6 were generally larger in size with
colonies of up to 50 cells or more readily detectable.
Similar augmentation was observed with a combination of
IL-3, G-CSF and IL-6. The enhancement of GM-CSF
5   dependent MK colony formation by IL-5, however, could
not be further augmented by IL-6. Time course studies
showed G-CSF to be most effective when added together
with GM-CSF or IL-3 at or shortly after the initiation
of culture, whereas IL-6 appeared to augment the G-CSF
10 induced enhancement when added as late as 7 days
provided that G-CSF was present at the initiation of
culture with GM-CSF or IL-3.

        As used herein the terms "G-CSF", "GM-CSF",
"IL-3", "IL-5", and "IL-6" denote proteins from natural
15 source extraction and purification, or from recombinant
cell culture systems. The terms likewise cover
biologically active equivalents; e.g. differing in one
or more amino acids in the overall sequence, or in
glycosylation patterns. Further the terms are intended
20 to cover substitution, deletion and insertion amino acid
variants, or past translational modifications.

        The subject invention also relates to
compositions which comprise, consist essentially of, or
consist of pharmaceutically effective amounts of G-CSF
25 and GM-CSF or IL-3, and optionally IL-6. In another
embodiment the invention relates to compositions which
comprise, consist essentially of or consist of
pharmaceutically effective amounts of GM-CSF and IL-5.

        The subject invention relates to methods for
30 enhancing thrombopoiesis. In order to analyze the
in vivo effects of IL-6 alone and in combination with
IL-3, GM-CSF and G-CSF on the hematopoietic system, the
cynomolgus monkey was selected as a model system based
upon similarities between simian and human
35 hematopoiesis. IL-6 alone or in combination with IL-3,
GM-CSF or G-CSF was administered on daily subcutaneous

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injections (10 µg/kg/d) over a varying time period.
When IL-6 alone was administered, peripheral blood
platelet counts increased two to three fold and reached
a maximum by day 10 of treatment.

5   The subject invention also relates to
compositions which comprise, consist essentially of, or
consist of pharmaceutically effective amounts of IL-6
and optionally IL-3 G-CSF or GM-CSF.

        Another embodiment of the subject invention is
10 the addition of stem cell factor to any of the
compositions or methods of treatment described herein.
Stem cell factor is described in commonly owned U.S.
Patent Application No. 422,383 hereby incorporated by
reference.

15   Also comprehended by the invention are
pharmaceutical compositions comprising pharmaceutically
effective amounts of the proteins noted above together
with suitable diluents, preservatives, solubilizers,
emulsifiers, adjuvants and/or carriers.

20   Several variables will be taken into account
by the ordinary artisan in determining the concentration
of the proteins in the therapeutic compositions, and the
dosages to be administered. Therapeutic variables also
include the administration route, and the clinical
25 condition of the patient.

        The methods and composition of the subject
invention are useful in treating thrombocytopenia, a
condition marked by a subnormal number of platelets in
the circulating blood and is the most common cause of
30 abnormal bleeding. Thrombocytopenia results from three
processes: (1) deficient platelet production,
(2) accelerated platelet destruction, and (3) abnormal
distribution of platelets within the body. A
compilation of specific disorders related to
35 thrombocytopenia is shown in Table A. Advantageous
applications of the subject invention are to

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thrombocytopenia resulting from deficient platelet
production and, in some cases, from accelerated platelet
destruction.

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TABLE A

Platelet Disorders

5   I.   Deficient Platelet Production

        A. Hypoplasia or suppression of megakaryocytes
            Chemical and physical agents (ionizing
            radiation, antineoplastic drugs), aplastic
10   anemia, congenital megakaryocytic hypoplasia
            myelophthisic processes, some viral infections
        B. Ineffective thrombopoiesis
            Disorders due to deficiency of vitamin B_{12} or
            folic acid
15   C. Disordered control mechanisms
            Deficiency of thrombopoietin, cyclic
            thrombocytopenia
        D. Miscellaneous
            Many hereditary forms

20
    II. Accelerated Platelet Destruction

        A. Due to immunologic processes
            Idiopathic Thrombocytopenia Purpura, drug-
25   induced antibodies, various hemolytic anemia,
            fetomaternal incompatability, post-
            transfusion.
        B. Due to nonimmunologic processes
            Kasabach-Merritt syndrome, thrombotic
30   thrombocytopenic purpura, infections (viral,
            bacterial, protozoan), massive transfusions

    III. Abnormal Platelet Distribution

35   A. Disorders of the spleen
        B. Hypothermia anesthesia

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Deficient platelet production commonly results
from hypoplasia or suppression of precursor
megakarocytes. Depletion of megakaryocyte pools can
occur during marrow injury caused by exposure to
5 myelosuppressive drugs or irradation. Thus patients
suffering from thrombocytopenia as a result of
chemotherapy or radiation therapy can be treated by
administration of pharmaceutically effective amounts of
GM-CSF or IL-3 in combination with pharmaceutically
10 effective amounts of G-CSF and IL-6 to raise blood
platelet counts and prevent bleeding disorders.
Depressed platelet levels may also result from
ineffective thrombopoiesis or disorders related to
thrombopietic control wherein megakaryocyte levels are
15 normal but maturation of megakaryocytes has been
disrupted. In these instances, an advantageous
treatment method would be the administration of a
pharmaceutically effective amount of IL-6 and optionally
a pharmaceutically effective amount of IL-3, GM-CSF or
20 G-CSF.

Accelerated platelet destruction can result in
thrombocytopenia even though the production of
megakaryocytes and platelets has not been diminished.
Disorders such as idiophathic thrombocytopenic purpura
25 (ITP) which are characterized by accelerated platelet
destruction mediated by an autoimmune response can be
treated by administration of immunosuppressants (such as
corticosteroids) combined with a pharmaceutically
effective amount of IL-6 and optionally a
30 pharmaceutically effective amount of IL-3, GM-CSF or
G-CSF.

GM-CSF and IL-3 play a primary role in the
in vitro colony formation of human CFU-MK. The results
presented below provide evidence for a regulatory role
35 for human G-CSF, IL-5 and IL-6 in the in vitro GM-CSF or
IL-3 dependent megakaryocyte colony formation. G-CSF,

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IL-5 and IL-6 increase cloning efficiency and colony
size. The ability of IL-6 to augment the G-CSF
enhancement of GM-CSF or IL-3 dependent MK colony
formation further corroborate the existence of
5   multifactorial as well as multi-level control mechanisms
in human megakaryocytopoiesis. The results from the
time course study below are suggestive of distinct
mechanisms of action for G-CSF and IL-6. G-CSF appears
to affect an early stage of megakaryocyte colony
10   formation, possibly the induction of the self renewal of
the MK precursors or the recruitment of earlier
progenitors to differentiate, while IL-6 appears to
affect a later event, perhaps by increasing colony size
or induction of commitment toward the MK lineage.
15

        The following examples are offered to more
fully illustrate the invention, but are not to be
construed as limiting the scope thereof.

20   EXAMPLE 1

Monoclonal antibodies and antisera

        Monoclonal anti-Leu 1, Leu 5b, Leu 4, Leu 11b,
25   Leu M1, Leu 16, Leu 19 were obtained from Becton
Dickinson (Mountain View, CA), OKT4, OKT9, OKB2 from
Ortho Diagnostic Systems (Raritan, NJ), My4 and Mol from
Coulter Immunology (Hialeah, FL), anti-glycophorin and
IOT8 from AMAC, Inc. (Westbrook, ME), monoclonal anti-
30   GPIIIa, anti-Factor VIII related antigen (FVIII) and B22
from Dako (Santa Barbara, CA), and GPIIb/IIIa were
obtained from Biodesign (Kennebunkport, ME). A sheep
anti-human albumin was purchased from Accurate Chemical
(San Diego, CA). An affinity purified goat anti-mouse
35   Ig and an alkaline phospatase conjugated $F(ab')_2$
fragment of affinity purified sheep anti-mouse IgG

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(containing 81.6 units of enzyme activity/ml) were
purchased from Cappel/Organon Teknika Corp.
(West Chester, PA). A sheep anti-human albumin was
purchased from Serotech (Accurate Chemical). Goat anti-
5   mouse IgG coated magnetic beads (Dynal) were purchased
from Robbin Scientific (Mountain View, CA).

EXAMPLE 2

10   Recombinant growth factors

        The specific activities of recombinant human
G-CSF, GM-CSF and IL-3 used in the subject experiments
were each approximately 10^8 U/mg as assayed according to
15   the procedure of Nicola et al. (26,27). Recombinant
human IL-5 and IL-6 showed specific activities of 10^6
U/mg, and $2x10^7$ U/mg respectively with a unit of
activity being defined as the reciprocal of the dilution
yielding half maximal activity in their respective assay
20   systems. IL-5 activity was assessed by eosinophil
differentiation from human marrow eosinophil progenitors
(28) and by the eosinophil peroxidase assay (29). IL-6
activity was assessed by measuring IgM production from
the human B lymphoblastoid cell line, SKW6-CL4 (30).
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EXAMPLE 3

Preparation of human bone marrow cells

30   Human bone marrow cells were obtained by iliac
crest aspiration from healthy volunteer donors after
informed consent. Buffy coat cells were separated from
marrow by centrifugation on Ficoll-paque (density
1.077g/ml; Pharmacia, Piscataway, NJ) at 500xg for
35   30 min. Inter-face cells were collected and washed with
phosphate buffered saline (PBS) containing 1% bovine

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serum albumin (BSA), and resuspended in Iscove's
modified Dulbecco's medium (IMDM) supplemented with 10%
heat inactivated fetal bovine serum (FBS). Marrow cells
were depleted of adherent cells by adherence to plastic
5 petri dishes for 90 min at 37°C in a humidified 5%
CO_2-95% air incubator. Unless otherwise stated, the
marrow nonadherent low density (NAL) cells were treated
with a monoclonal antibody mixture to deplete T cells
(anti Leu 1, Leu 5b, OKT4, IOT8), B cells (anti-CD22,
10 OKB2), NK cells (Leu 11b, Leu 19), residual monocytes,
granulocytes and other differentiated myeloid cells (Leu
M1, Mol, My4), erythroid elements (anti-glycophorin) and
activated and proliferating cells (OKT9). In some
experiments, only T cell depletion was carried out on
15 the NAL cells. This was done by treatment with
anti-Leu 1. Depletion of monoclonal antibody bound
cells was accomplished by panning of goat anti-mouse IgG
(50 μg/dish) coated petri dishes (Bectodishes, 100x15mm,
Fisher Scientific), followed by complement mediated
20 lysis with 1:15 dilution of a Low-Tox rabbit complement
(Accurate Chemical, Westbury, NY) (31). To ensure
exhaustive depletion of the antibody bound cells, the
cell suspensions were further treated with 100 μl
anti-Ig coated magnetic beads per 10^6 cells for 20 min
25 and the bound cells were removed by a magnet (32). The
resultant populations were referred to as accessory
depleted bone marrow cells (Acc⁻BMC) OR T depleted bone
marrow cells (NALT-BMC). NALT-BMC constituted
approximately 40% of the starting population, whereas
30 Acc-BMC was approximately 5% of the starting population.

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EXAMPLE 4

Clonal growth of megakaryocyte progenitors (CFU-MK) in
agarose

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        Unless otherwise stated, Acc-BMC at 10^4 cells
per plate were cultured in 35mm Lux petri dishes
(Nunc, Inc.) in triplicates in 1ml of 0.32% agarose in
IMDM medium supplemented with 10% heat inactivated fetal
10 bovine serum (Hyclone). Cultures were incubated at 37°C
in a $5\%CO_2$-95% air humidified incubator for 12 days.

EXAMPLE 5

15 Identification of megakaryocyte colonies

        Megakaryocyte colonies were detected by a
modification of the immuno-alkaline phosphatase
technique described by Hanson et al. (34) using
20 monoclonal antibodies against platelet glycoprotein
GPIIIa or GPIIb/IIIa or factor VIII and alkaline
phosphatase labeled $F(ab')_2$ fragment of the affinity
purified sheep anti-mouse IgG (AP-anti-Ig). All
antisera and monoclonal antibodies were diluted in PBS +
25 1% bovine serum albumin (BSA). Culture plates were
fixed with 2.5% formalin in acetone for 1 min, washed
and transferred to Corning 75x50mm microslides and air
dried. The dried slides were soaked in water for 5-10
min to remove salts and color from the indicator present
30 in culture medium. The slides were then air dried
overnight, treated with PBS containing a 2% solution of
human AB serum and 2% lamb serum to block nonspecific
binding sites, followed by a 1:150 dilution of
anti-GPIIIa or GPIIb/IIIa or anti-FVIII for 1hr at 4°C,
35 washed for 1hr in a 1:1 diluted PBS. The slides were
then treated for an hour at 4°C with AP-anti-Ig at 1:250

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dilution, followed by extensive washing with 1:1 diluted
PBS to remove excess unbound antibodies. Controls were
slides not treated with antibodies, or treated with the
blocking antibody followed by AP-anti-Ig. Treated and
5 control slides were then developed for 20-30 min in a
freshly prepared mixture of 0.26mg/ml α-naphthol AS-BI
phosphate (solubilized in dimethyl formamide) and 1mg/ml
Fast Red TR salt in O.2M Tris buffer (pH 9.0) containing
5mM $MgCl_2$ and 1mM Levamisole (all from Sigma) as an
10 inhibitor of cellular alkaline phophatase and counter
stained for 5 min with Mayer's hematoxylin (Sigma).
Megakaryocyte colonies positive for GPIIIa or FVIII show
reddish cytoplasmic staining. Colonies on untreated and
AP-anti-Ig treated slides gave no staining or weak
15 background staining.

        Results of triplicate cultures from single or
multiple experiments were expressed as mean ± standard
deviation (x ± S.D.). Statistical significance where
indicated was determined using a two-tailed Student's
20 t-test.

                        EXAMPLE 6

Enrichment of megakaryocyte progenitors by depletion of
25 accessory cells

        The ability of NALT⁻ and Acc⁻BMC to form
megakaryocyte colonies in the presence of 1000U/ml
GM-CSF was assessed. The removal of T cells or
30 accessory cells did not impair CFU-MK colony formation
(Table 1).

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TABLE 1

Enrichment of CFU-MK in marrow depleted of accessory cells

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Treatment of BMC	No. Cells Plated	No. CFU-MK ± SD*	Cloning efficiency(%)
NAL BMC	10^5	27 ± 2	0.03 ± 0.002
		29 ± 1	0.03 ± 0.001
NALT⁻ BMC	$5x10^4$	46 ± 6	0.09 ± 0.012
		25 ± 11	0.05 ± 0.022
		53 ± 0	0.11 ± 0.000
Acc⁻ BMC	10^4	46 ± 3	0.46 ± 0.03+
		22 ± 2	0.22 ± 0.02+
		25 ± 4	0.25 ± 0.04+
		34 ± 6	0.34 ± 0.06+

* All cultures contain 10 ng (1000U) recombinant human GM-CSF
20 per plate. Data represent the mean colony counts from
triplicate cultures ± standard deviation. Results from separate
experiments are listed.
+ Statistically significant compared to NAL MBC, $p < 0.05$.

25 T depletion which removed approximately 40% of marrow
nucleated cells resulted in approximately a two fold
increase in cloning efficiency, whereas accessory
depletion which removed as much as 95% of the marrow
nucleated cells resulted in a 10 fold or more increase
30 in cloning efficiency.
        A comparison of various cytokines showed that
both GM-CSF and IL-3 possess MK colony stimulating
activity, while G-CSF, IL-5 and IL-6 were inactive
(Table 2).

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TABLE 2

Effect of various recombinant hemopoietic factors on in vitro
megakaryocyte colony formation*

Factor Added	Concentration ng/ml	No. Colonies ± SD (n) +	
		Pool 1	Pool 2
None (Medium Control)	---	None	None
GM-CSF	10	24 ± 3(3)	49 ± 6 (2)
	1	14 ± 2(2)	40 ± 10(10)
	0.1	ND	46 ± 6 (2)
	0.01	ND	10 ± 0 (1)
IL-3	100	12 ± 2(1)	50 ± 10(1)
	10	14 ± 3(2)	40 ± 5 (1)
	1	ND	20 ± 5 (1)
G-CSF	1 - 10	None (2)	
IL-5	1 - 100	None (2)	
IL-6	1 - 10	None (2)	

* Accessory depleted bone marrow cells were plated at 10^4 cells/ml.

+ Results are expressed as mean colony number ± standard deviation
(SD). n represents the number of experiments pooled. Some donor
cells showed significantly higher response than others, hence these
were pooled separately. ND, not determined.

G-CSF stimulated mainly neutrophil colonies, and IL-5
stimulated small numbers (generally less than 10)
colonies of eosinophils (Luxol fast blue positive)
whereas IL-6 at the concentrations tested had no
detectable colony stimulating activity. GM-CSF

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stimulated MK colonies were generally of small size,
mostly containing less than 10 cells per colony. IL-3
stimulated colonies tend to be slightly larger (5-20
cells). Immuno-alkaline phosphatase staining followed
5   by Hematoxylin counter staining showed these MK colonies
to be strongly positive for GPIIIa as well as FVIII and
to have relatively high nuclear to cytoplasmic ratio
(Plate 1). Control slides and non-MK colonies on the
same slides showed no or very weak alkaline phosphatase
10 staining (Plate 1). Concentrations of GM-CSF and IL-3
as low as 10 and 100 U/ml respectively were found to be
adequate in stimulating plateau levels of MK colony
growth As different donors were used for each
experiment, some differences in cloning efficiency among
15 donors were observed. Some showed plateau levels of
$10-20$ colonies/10^4 Acc⁻BMC, while others were
significantly higher (40-50 colonies). Hence, for
clarity, the data were pooled separately (Pools 1&2,
Table 2).
20

EXAMPLE 7

Enhancement of MK colony formation by G-CSF and IL-5

25   Although G-CSF by itself was inactive as a MK
colony stimulating factor, it was nevertheless able to
enhance the GM-CSF or IL-3 stimulated MK colony
formation (Table 3).

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TABLE 3

Enhancement of GM-CSF and IL-3 induced
megakaryocyte colony formation by G-CSF and IL-5

Factor(s) Added	No. Colonies ± SD*			
	Exp 1	Exp 2	Exp 3	Exp 4
GM-CSF (1ng/ml)	8 ± 3	13 ± 2	55 ± 1	149 ± 20
GM-CSF (1ng/ml) + G-CSF(0.01-1ng/ml)	27 ± 7+	21 ± 2+	ND	199 ± 23+
GM-CSF (1ng/ml) + IL-5(1-10ng/ml)	44 ± 0+	26 ± 3+	ND	ND
GM-CSF (1ng/ml) + IL-6(1-10ng/ml)	7 ± 1	14 ± 6	43 ± 4	ND
IL-3 (10ng/ml)			40 ± 5	
IL-3 (10ng/ml) + G-CSF(0.1-1ng/ml)			76 ± 7+	
IL-3 (10ng/ml) + IL-6(10ng/ml)			48 ± 13	
G-CSF (1ng/ml) + IL-6(10ng/ml)		None	None	
IL-5 (10ng/ml) + IL-6(10ng/ml)		None	None	

* Accessory depleted bone marrow cells were plated at 10^4
cells/ml. Results from separate experiments are expressed as
mean colony counts of triplicate cultures ± standard
deviation. In cases where a range of concentrations of G-CSF,
IL-5 or IL-6 were indicated, the values shown represents the
group means and standard deviations.

+ Statistically significant compared to GM-CSF or IL-3 controls,
p<0.05.

This enhancement could be detected irrespective of the
baseline level of MK colony response to GM-CSF or
IL-3. Thus, marrow cells that showed low or high
numbers of MK colonies with GM-CSF or IL-3 showed
corresponding enhancement with the addition of G-CSF

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(N.B. The high number of MK colonies with GM-CSF in
Exp.4 was an isolated occurrence). In some
experiments, the effect of IL-5 and IL-6 on GM-CSF or
IL-3 dependent MK colony formation was also

5   analyzed. Like G-CSF, IL-5 was found to enhance
GM-CSF induced megakaryocytopoiesis. IL-6, on the
other hand, was without direct enhancing effect on
either GM-CSF or IL-3 dependent MK colony formation.
Moreover, the enhancement by G-CSF and IL-5 was not

10 limited to colony number. An increase in the
frequency of colonies with 10 to 50 cells was also
observed (Table 4).

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TABLE 4

Effect of growth factors on megakaryocyte colony size
distribution

Growth Factor(s) Added	Percent* (x ± SD) of colonies with		
	3-10 cells	10-50 cells	>50 cells
None	0	0	0
GM-CSF	97 ± 4	1 ± 1	0
GM-CSF + IL-5	80 ± 3+	20 ± 3+	0
GM-CSF + G-CSF	69 ± 6+	31 ± 6+	0
GM-CSF + G-CSF + IL-6	29 ± 9+	68 ± 9+	3 ± 1
IL-3	83 ± 1	16 ± 1	1 ± 1
IL-3 + G-CSF	71 ± 13	27 ± 13	2 ± 1
IL-3 + G-CSF + IL-6	40 ± 1	49 ± 12+	11 ± 8+

* The percentages were obtained by counting all MK colonies on
one plate. Data of randomly selected plates of each group from
2-4 experiments were pooled. GM-CSF, IL-3 and G-CSF were all
used at 1 ng/ml while IL-5 and IL-6 were tested at 10 ng/ml in
these experiments.

+ Statistically significant when compared to GM-CSF or IL-3
controls, p<0.05.

EXAMPLE 8

Augmentation of G-CSF enhancement of MK colony formation
by IL-6.

While IL-6 failed both to stimulate MK colony
formation directly and to enhance GM-CSF and IL-3
stimulated MK colony formation, it nevertheless was able
to further augment the G-CSF enhancement of MK colony
formation (Fig. 1 & 2). Thus, when IL-6 was added to
cultures containing GM-CSF and G-CSF or IL-3 and G-CSF,
a further increase in the number of MK colonies was
observed. MK colony size was also increased, with the
appearance of more MK colonies consisting of up to
50 cells or more (Table 4 and Plate 2a-f). This augm-
ntation by IL-6 was specific for the G-CSF induced
enhancement and its effect was not replacible with IL-5.

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EXAMPLE 9

Time course of action of G-CSF and IL-6 augmentation of
GM-CSF or IL-3 induction of MK colony formation.

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    To further analyse if G-CSF and IL-6 affect MK
colony formation at the same stage or at different
stages, a time course study was carried out. As can be
seen in Table 5, the G-CSF enhancement of GM-CSF or IL-3
10 induced MK colony formation was optimal when both
factors were present early on (no later than day 3) in
the culture period.

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Table 5
Time Course of Effect of G-CSF and IL-6

	Time of Addition of		No. CFU-Mk per 10^4 Acc-BMC		
GM-CSF/IL-3	G-CSF	IL-6	Exp. 1	Exp. 2	Exp. 3§
d0	None	None	9±2	30±4	17±5
d0	d0	None	19±3*	74±4*	49±6*
d0	d3	None	15±6	75±9*	44±5*
d0	d5	None	15±5	69±27	39±8*
d0	d7	None	7±1	37±6	27±1
d0	d0	d0	29±3+	134±6+	74±4+
d0	d3	d3	31±4	130±8+	62±2
d0	d5	d5	6±2	35±0	29±1
d0	d7	d7	3±1	39±5	21±6
d0	d0	d3	32±5+	102±7+	76±6+
d0	d0	d5	27±7	99±10+	83±10+
d0	d0	d7	33±3+	91±4+	92±0+

§ In Exp. 3, IL-3 was used instead of GM-CSF, GM-CSF, G-CSF and IL-3 were all used at 1 ng/ml, whereas, IL-6 was used at 10 ng/ml in these experiments.
*Statistically significantly different (p<0.05) when compared to results of GM-CSF or IL-3 alone.
+ Statistically significantly different from the corresponding results with Gm-CSF+G-CSF or Il-3+G-CSF.

Likewise, the synergistic effect of G-CSF and IL-6 was observed only when both factors were added together with GM-CSF or IL-3 at the initiation of culture or no later than 3 days afterwards. However, when G-CSF was present at the initiation of culture with GM-CSF or IL-3, then the IL-6 induced augmentation could be observed even when IL-6 was added as late as 5-7 days.

EXAMPLE 10

In vivo effects of IL-6 on hematopoiesis.

Juvenile healthy female cynomolgus monkeys (Macaque fasicularis) were obtained from

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Tierlaboratorium, Universitat Dusseldorf, FRG. They
were maintained according to the German guidelines for
the use and care of laboratory animals. Blood samples
were taken by puncture of peripheral veins. Prior to
5   all manipulations, animals were anesthetized with
ketamine hydrochloride (Hersteller).

Hematologic counts were performed by an
automated hematologic analyzer. Differential counts of
leukocytes were performed on slides stained with May-
10 Gruenwald-Giemsa.

To assess the biological activity of IL-6 on
platelet production, daily subcutaneous injections of
IL-6 at 10 ug/kg/d were given to three healthy
monkeys. The peripheral blood platelet counts were
15 measured daily and are shown in Figure 3. Animal #1
received IL-6 from day 1 to day 18, animal #2 and #3
from day 1 to day 21. In all three monkeys the platelet
counts (cells per ul) increased significantly, in animal
#1 from 254,000 to 756,000; in animal #2 from 513,000 to
20 786,000; in animal #3 from 276,000 to 592,000. IL-6 did
not significantly change the number of peripheral blood
neutrophils, eosinophils, basophils, lymphocytes and
erythrocytes.

IL-6 and IL-3 were administered simultaneously
25 as daily subcutaneous injections to one monkey,
10 ug/kg/d each, to investigate the in vivo effects of
this combination. Levels of peripheral blood platelets
in animal #4, which had been treated from day 1 to
day 32, are shown in Figure 4. In combination with
30 IL-3, IL-6 led to an increase of peripheral blood
platelets from 362,000 to a maximum of 675,000 on day 10
of the treatment. This increase is equivalent to that
seen with IL-6 alone. The combination of IL-6 and IL-3
significantly increased eosinophil counts from 214 to a
35 maximum of 4,089 and basophil counts from initially 0 to
a maximum of 2,240. Levels of other blood cells, such

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as neutrophils, monocytes, lymphocytes and erythrocytes did not change significantly.

Two monkeys were treated with the combination of IL-6 and G-CSF, each 10 ug/kg/d subcutaneously, over

5 a time period of 17 days in animal #5 and 25 days in animal #6. In animal #5 the peripheral blood platelet counts increased from 252,000 to a maximum of 638,000; in animal #6 from 342,000 to a maximum of 1,104,000. These results are shown in Figure 5. Neutrophils

10 increased from 1,624 to a maximum of 33,947 (for animal #5) and from 5,060 to 52,809 (for animal 6). Lymphocytes increased from 3,864 on day 1 to a maximum of 9,693 (for animal #5) and from 5,500 to a maximum of 16,628 (for animal #6). There was no significant change

15 in the levels of the other peripheral blood cells.

Animal #7 has been treated with a combination of IL-6 and GM-CSF as described above from day 1 to day 21. Platelet counts increased from 334,000 initially to a maximum of 538,000 as shown in Figure 6. Neutrophil

20 counts rose significantly from 2,652 to a maximum of 34,286, eosinophil counts increased from 156 to 4,704, and lymphocyte counts increased from 12,480 to 16,800. There was no significant change in the levels of basophils and monocytes.

25

*   *   *

While the invention has been described in what

30 is considered to be its preferred embodiments, it is not to be limited to the disclosed embodiments, but on the contrary, is intended to cover various modifications and equivalents included within the spirit and scope of the appended claims, which scope is to be accorded the

35 broadest interpretation so as to encompass all such modifications and equivalents.

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WHAT IS CLAIMED IS:

1. A method of increasing megakaryocyte
production in a mammal comprising administering a
5   pharmaceutically effective amount of G-CSF and a
pharmaceutically effective amount of IL-3 or GM-CSF.

2. A method as in Claim 1 wherein the G-CSF
is administered simultaneously with GM-CSF or IL-3
10   administration or after GM-CSF or IL-3 administration.

3. A method as in Claim 1 further comprising
administering a pharmaceutically effective amount of
IL-6.
15

4. A method as in Claim 3 wherein the IL-6 is
administered from 0-7 days after the G-CSF
administration.

20   5. A method of increasing megakaryocyte
production comprising administering a pharmaceutically
effective amount of IL-5 and a pharmaceutically
effective amount of GM-CSF.

25   6. A method as in Claim 5 wherein the IL-5 is
administered simultaneously with GM-CSF administration
or just after GM-CSF administration.

7. A method of increasing production of blood
30   platelets in a mammal comprising administering a
pharmaceutically effective amount of IL-6.

8. A method as in Claim 7 further comprising
administering a pharmaceutically effective amount of
35   IL-3, G-CSF or GM-CSF.

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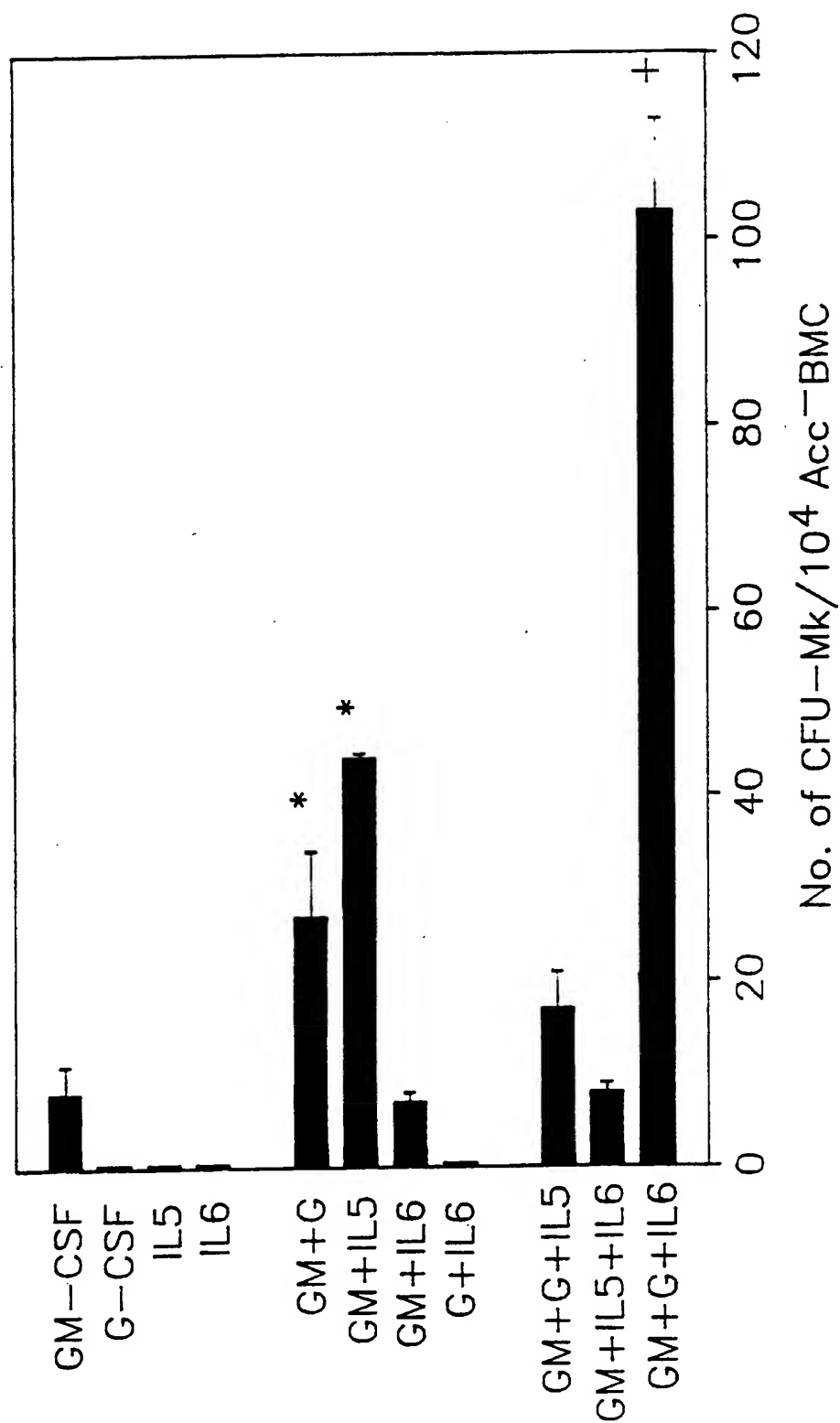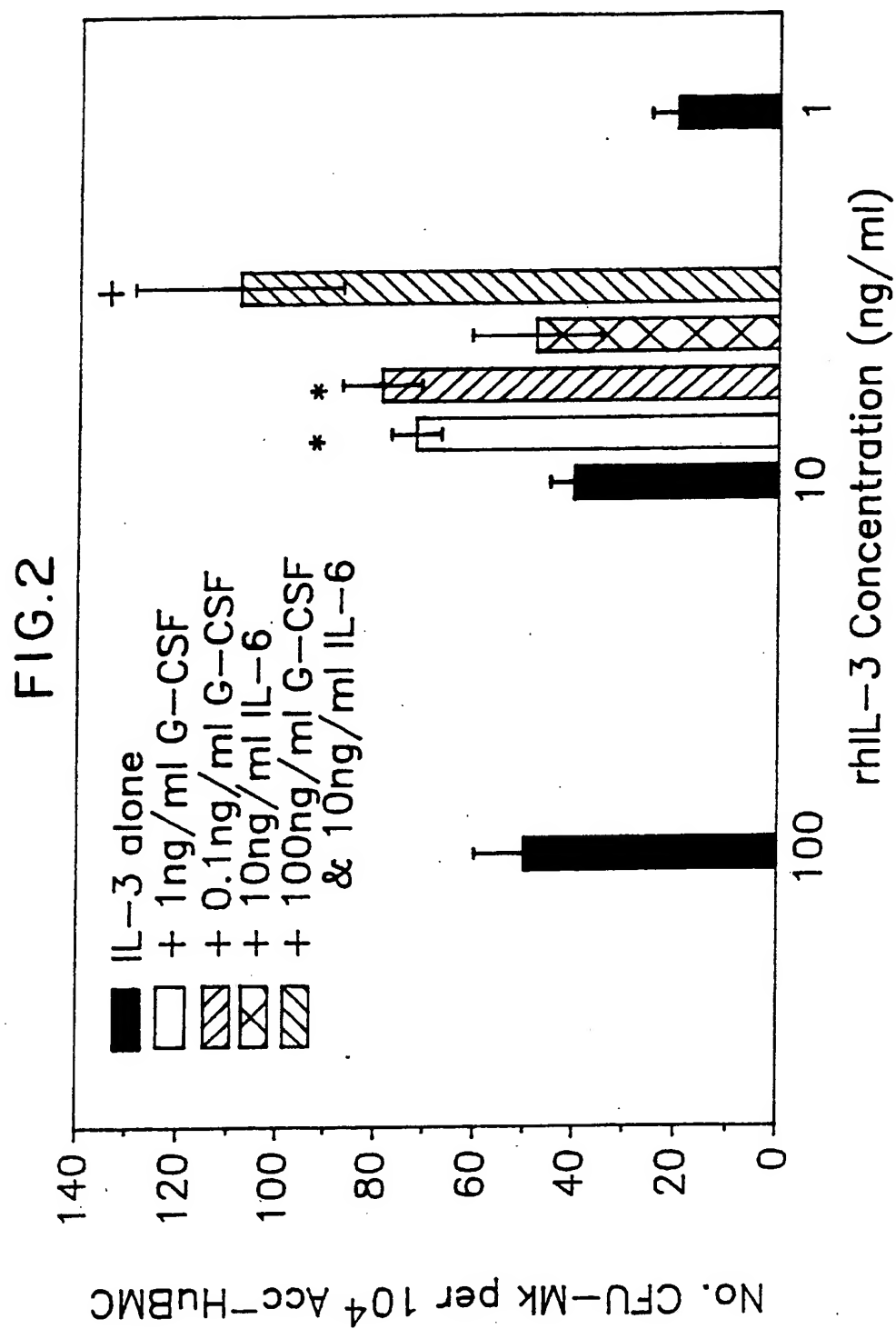9.   A composition comprising a pharmaceutically effective amount of G-CSF and a pharmaceutically effective amount of IL-3 or GM-CSF.

5   10.   A composition as in Claim 9 further comprising a pharmaceutically effective amount of IL-6.

11.   A composition comprising a pharmaceutically effective amount of IL-5 and a
10   pharmaceutically effective amount of GM-CSF.

12.   A composition comprising a pharmaceutically effective amount of IL-6 and a pharmaceutically effective amount of IL-3, G-CSF or
15   GM-CSF.

13.   A composition as in Claims 10, 11 or 12 further comprising a pharmaceutically acceptable diluent, adjuvant, preservative, stabilizer, emulsifier
20   and/or carrier.

14.   A kit comprising a vial containing a pharmaceutically effective amount of G-CSF and a vial containing a pharmaceutically effective amount of IL-3
25   or GM-CSF.

15.   A kit as in Claim 11 and further comprising a vial containing a pharmaceutically effective amount of IL-6.

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16.   A kit comprising a vial containing a pharmaceutically effective amount of IL-5 and a vial containing a pharmaceutically effective amount of GM-CSF.

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17. A kit comprising a vial containing a pharmaceutically effective amount of IL-6 and a vial containing a pharmaceutically effective amount of IL-3, G-CSF or GM-CSF.

5

18. A method for the treatment of thrombocytopenia in a mammal caused by deficient platelet production comprising administering a pharmaceutically effective amount of G-CSF and a
10   pharmaceutically effective amount of IL-3 or GM-CSF.

19. A method as in Claim 18 wherein thrombocytopenia results from chemotherapy.

15   20. A method as in Claim 18 wherein thrombocytopenia results from radiation therapy.

21. A method as in Claim 18 wherein G-CSF is administered simultaneously with GM-CSF or IL-3
20   administration or after GM-CSF or IL-3 administration.

22. A method as in Claim 18 further comprising administering a pharmaceutically effective amount of IL-6.

25

23. A method as in Claim 18 wherein IL-6 is administered from 0-7 days after G-CSF administration.

24. A method for the treatment of
30   thrombocytopenia in a mammal caused by accelerated platelet destruction or by deficient platelet production comprising administering a pharmaceutically effective amount of IL-6.

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25. A method as in Claim 24 wherein
thrombocytopenia results from idiopathic
thrombocytopenia purpura.

5   26. A method as in Claim 24 further
comprising administering pharmaceutically effective
amount of IL-3, G-CSF or GM-CSF.

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FIG.1

FIG.2

IL-3 alone
+ 1ng/ml G-CSF
+ 0.1ng/ml G-CSF
+ 10ng/ml IL-6
+ 100ng/ml G-CSF
& 10ng/ml IL-6

rhIL-3 Concentration (ng/ml)

No. CFU-Mk per 10⁴ Acc-HuBMC

FIG.3A
INTERLEUKIN-6 TREATMENT

Platelets

animal #1

10 µg/kg/d IL-6

days

$x \cdot 10^3/\mu l$
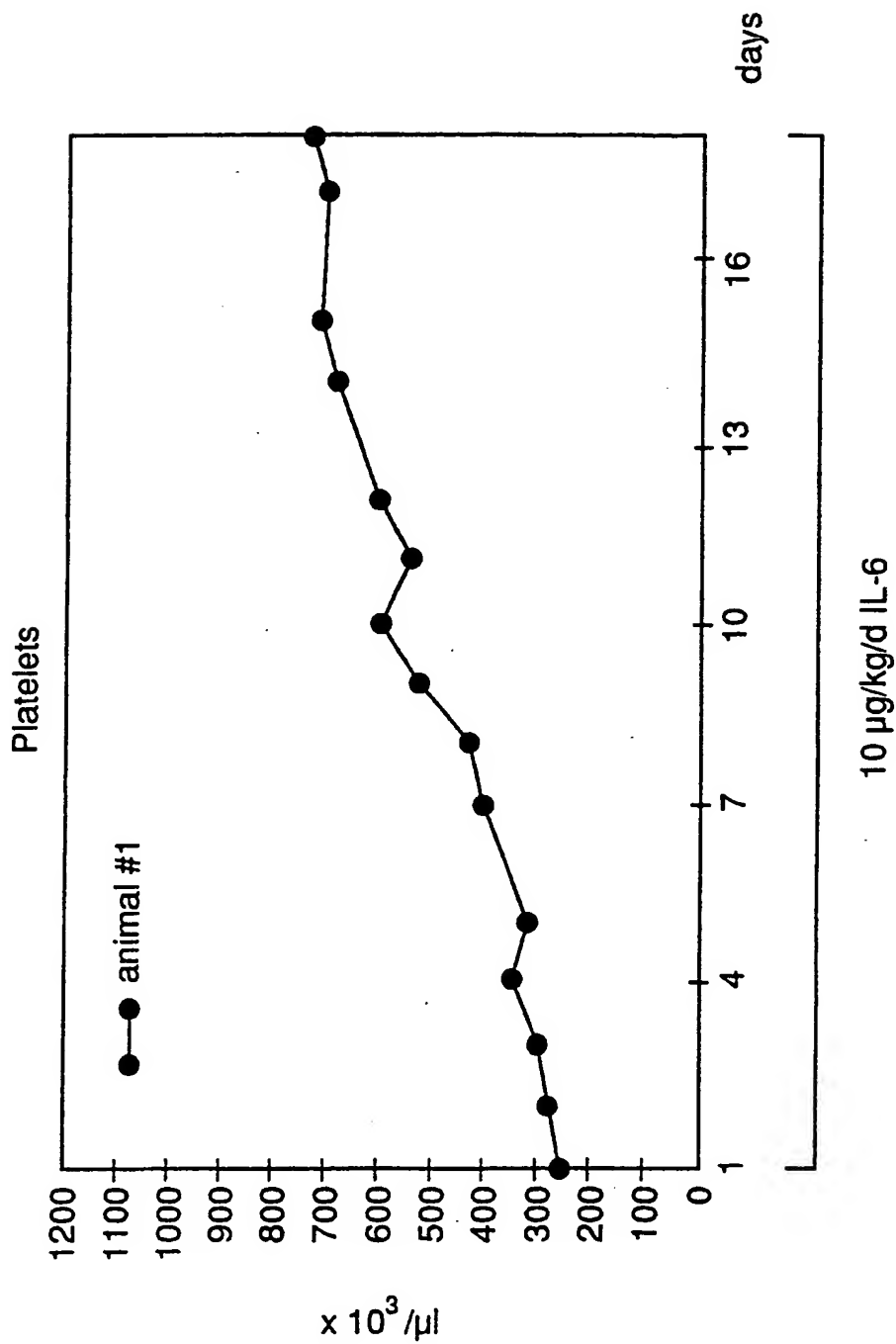
FIG.3B
INTERLEUKIN-6 TREATMENT
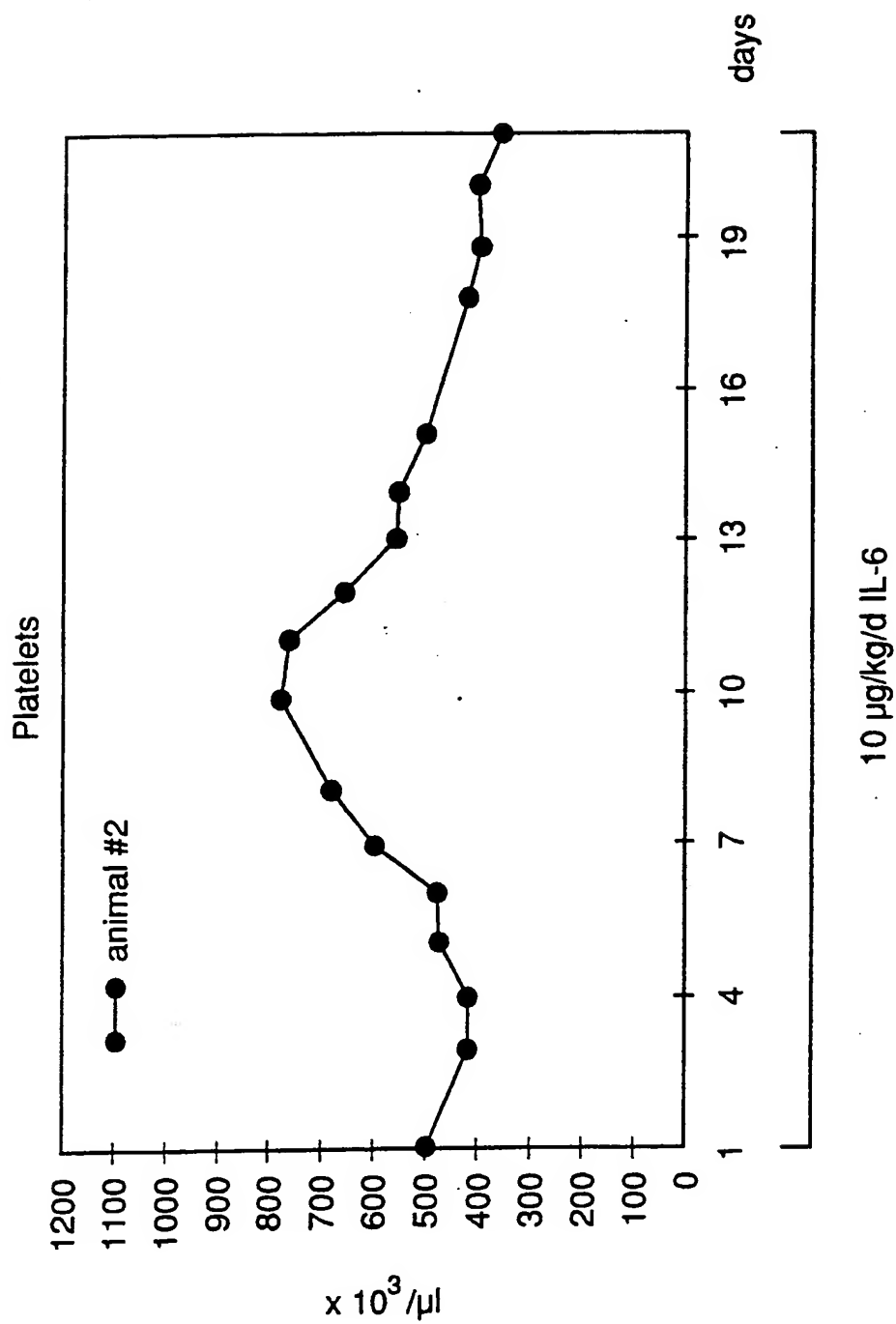
FIG.3C
INTERLEUKIN-6 TREATMENT

Platelets

animal #3

x 10³/µl

days

10 µg/kg/d IL-6
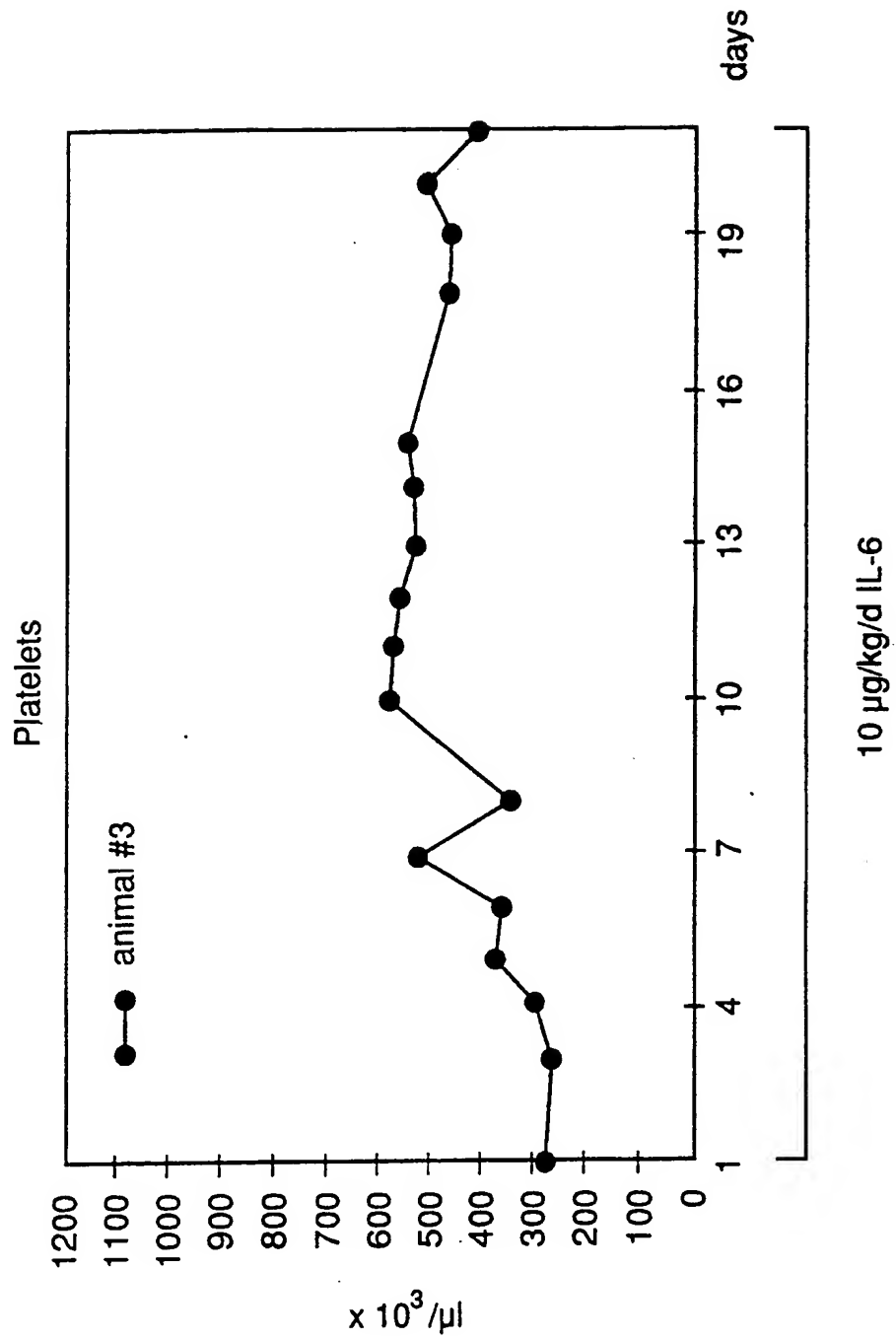
FIG. 4
COMBINATION OF IL-6 WITH IL-3
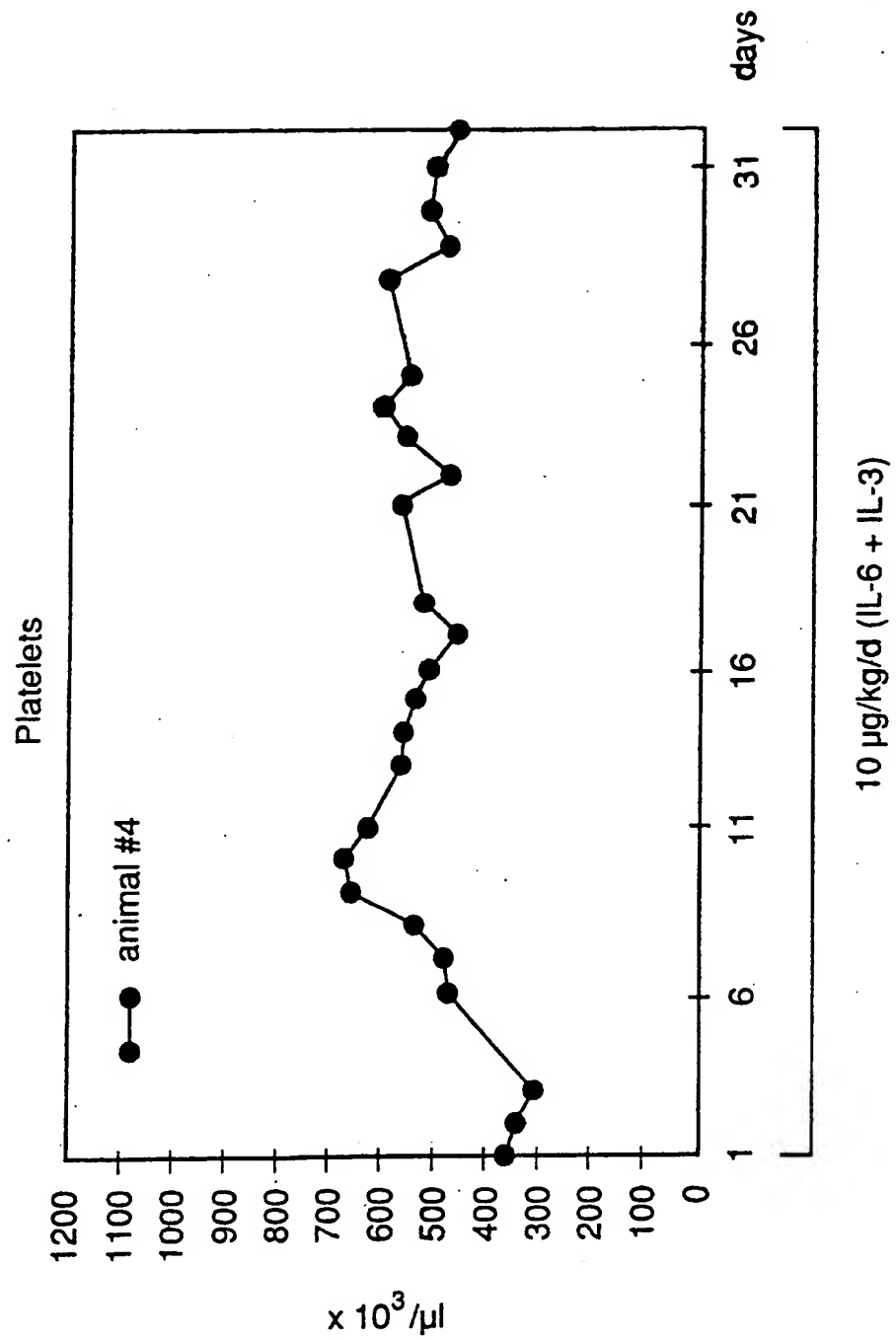
FIG.5
COMBINATION OF IL-6 WITH G-CSF

Platelets

10 μg/kg/d (IL-6 + G-CSF)

- ●—● animal #5
- ●┈┈● animal #6

x 10³/μl

days

FIG.6
COMBINATION OF IL-6 WITH GM-CSF

Platelets

animal #7

10 µg/kg/d (IL-6 + GM-CSF)
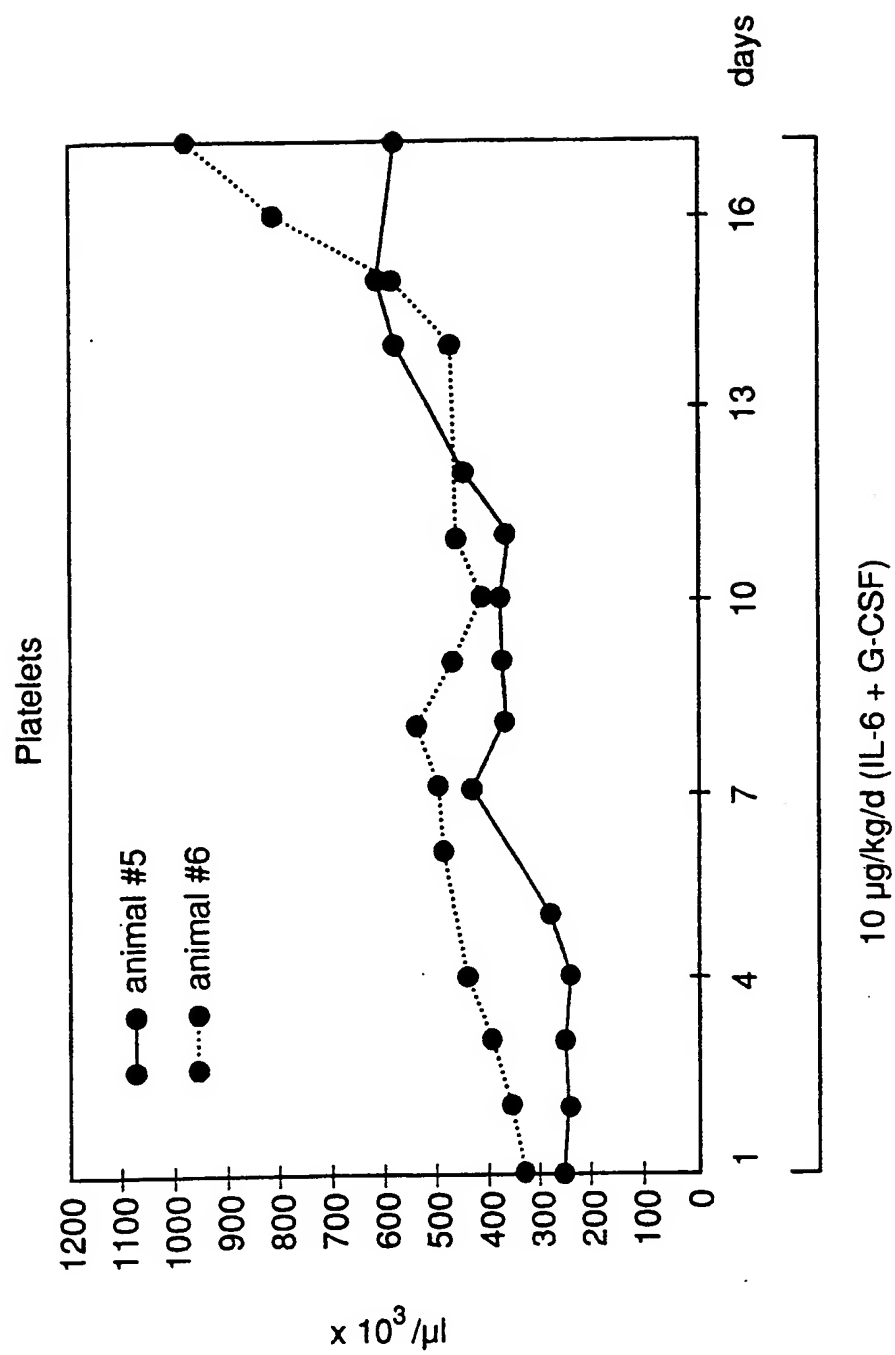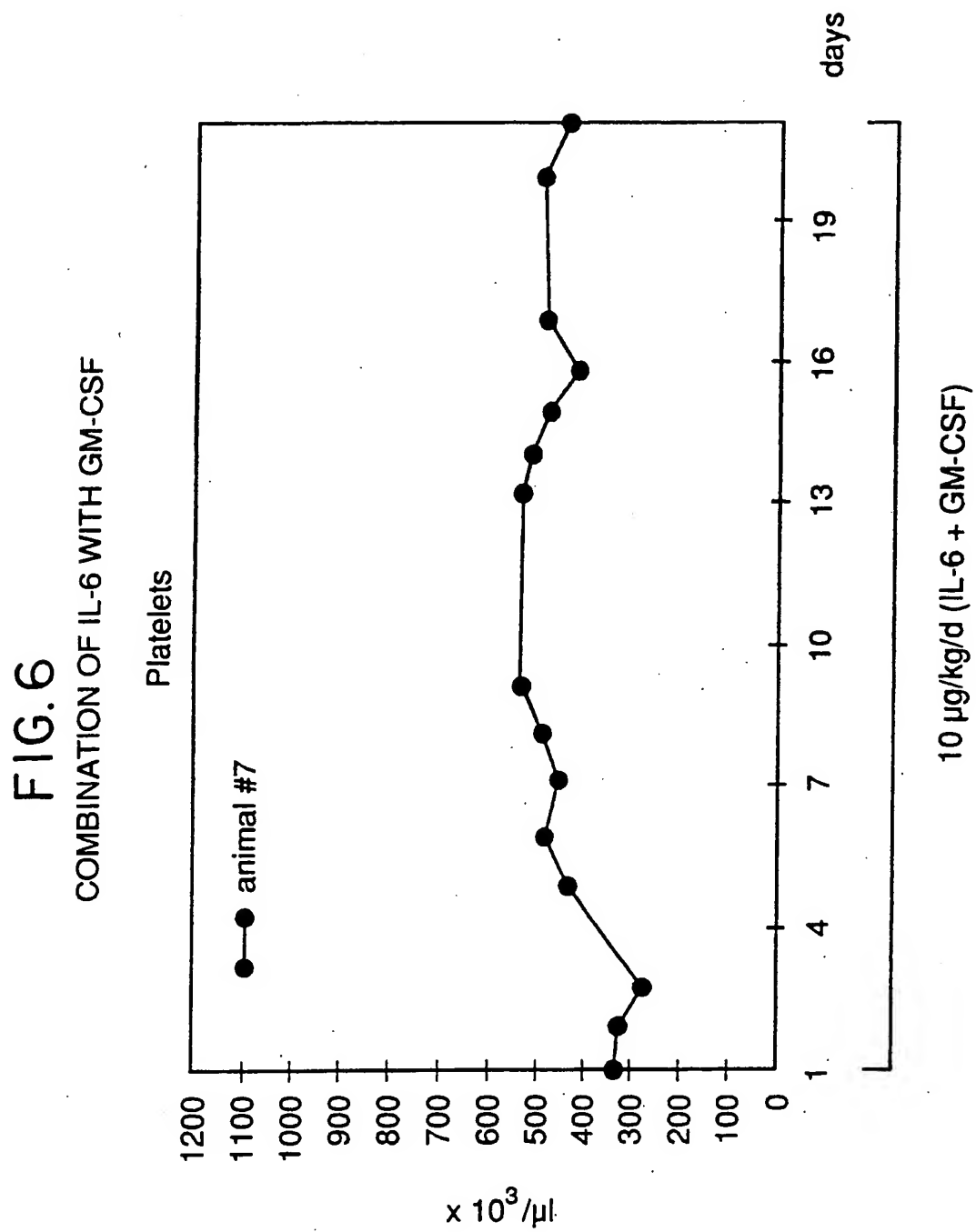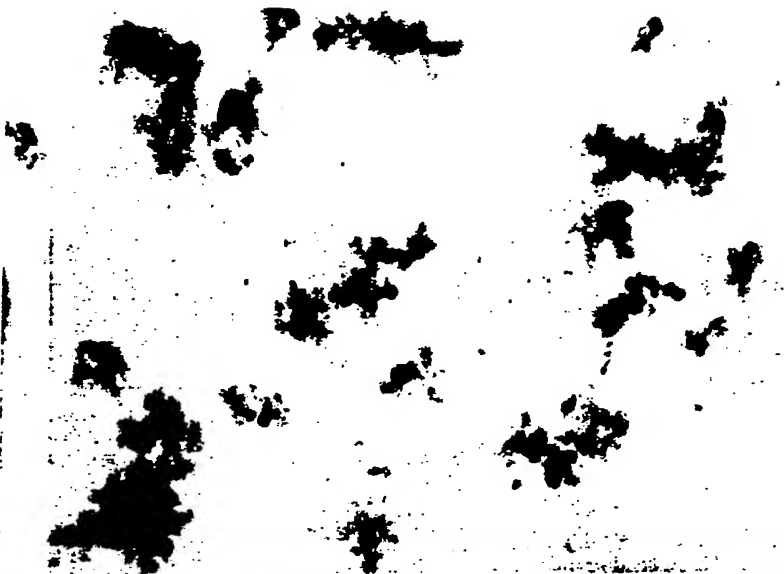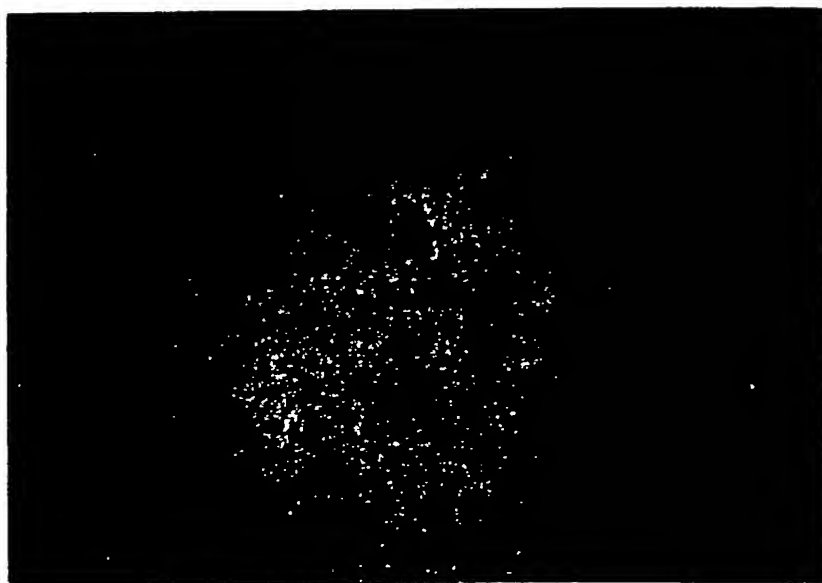
PLATE 1A

PLATE 1B

PLATE 1C
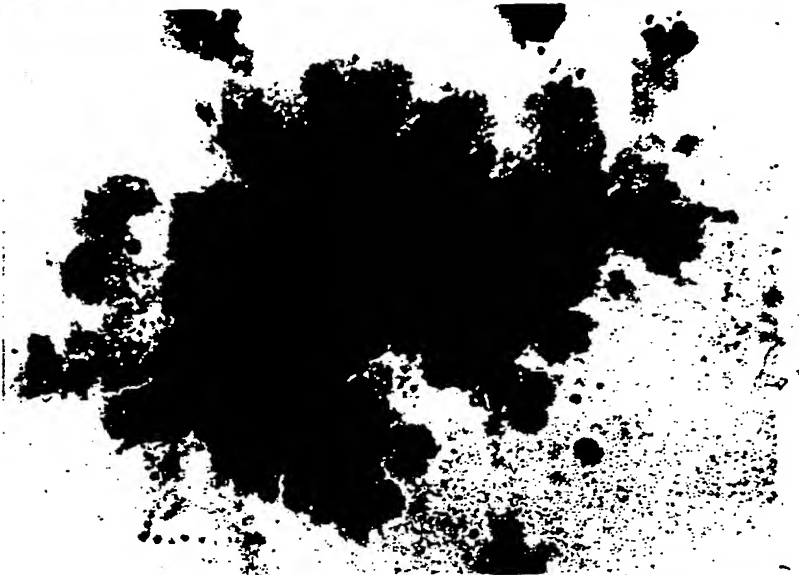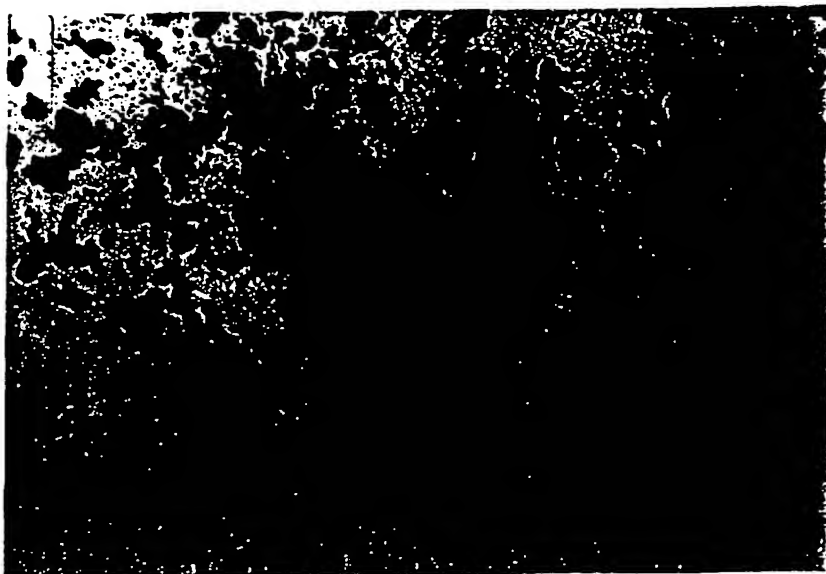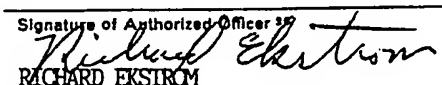
PLATE 2A

PLATE 2B

PLATE 2C

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(S): A61K 45/05, 37/10
U.S. Cl.: 424/85.1, 85.2; 514/8

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S. Cl.	424/85.1, 85.2; 514/8

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [5]

MEDLINE, search terms: IL3, interleukin-3, IL5, interleukin-5, IL6, interleukin-6, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, megakaryocyte, platelet, thrombocyte

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category [*]	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X, P Y	US,A, 4,962,091 (Eppstein et al.) 09 October 1990, see column 7 and the claims.	18-26 1-17
X Y	US,A, 4,879,111 (Chong) 07 November 1989, see column 4.	18-26 1-17
X Y	US,A, 4,604,377 (Fernandes et al.) 05 August 1986, see column 9.	18-26 1-17
X	US,A, 4,512,971 (Wissler et al.) 23 April 1985, see entire document.	1-26
X	US,A, 4,468,379 (Gottlieb) 28 August 1984, see entire document.	1-26

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
06 MARCH 1990	15 APR 1991
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	RICHARD EKSTROM

Form PCT/ISA/210 (second sheet) (May 1986)

	FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET	
x,p	European Journal of Immunology, Volume 20, No.9, issued September 1990, H. Kimura et al., "Interleukin 6 is a differentiation factor for human megakaryocytes in vitro", pages 1927-1931, see the abstract.	3-4,7-8,10,12-13 15,17,22-26
x,p	The Journal of Immunology, Volume 144, No.4, issued 15 February 1990, W.E. Secor et al., "Eosinophils and immune mechanisms. VI. The synergistic combination of granulocyte-macrophage colony stimulating factor and IL-5 accounts for eosinophil-stimulation promoter activity in Schistosoma mansoni-infected mice", pages 1484-1489, see entire document.	5,6,11,13,15,16

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers .... , because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers .... , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____ , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

SEE ATTACHMENT

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2) (Rev. 4-90)

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No 18
X	The Journal of Immunology, Volume 140, No.1, issued 01 January 1988, D.J. Warren et al., "Synergism among interleukin 1, interleukin 3, and interleukin 5 in the production of eosinophils from primitive hemopoietic stem cells", pages 94-99, see the abstract.	5,6,11,13 15,16
X,P	Experimental Hematology, Volume 17, No.11, issued December 1989, M.K. Warren et al., "The role of interleukin 6 and interleukin 1 in megakaryocyte development", pages 1095-1099, see the abstract.	3,4,7,8,10 12,13,15 17,22-26
X	Experimental Hematology, Volume 17, No.10, issued November 1989, E. Bruno et al., "Effect of interleukin 6 on in vitro human megakaryocytopoiesis: Its interaction with other cytokine", pages 1038-1043, see the abstract.	3,4,7,8,10 12,13,15, 17,22-26
X / Y	Experimental Hematology, Volume 17, No.8, issued September 1989, S. Tanikawa et al., "Effects of recombinant granulocyte colony stimulating factor (rG-CSF) and recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF) on acute radiation hematopoietic injury in mice", pages 883-888 see the abstract and pages 884-885.	3-6,8,10- 12,15-17 19,22-23 1,2,9,13, 14,18,20 21
X,P	Blood, Volume 75, No.12, issued 15 June 1990, K. Koike et al., " Interleukin-6 enhances murine megakaryocytopoiesis in serum-free culture", pages 2286-2291, see the abstract.	3,4,7,8,10 12,13,15,17 22-26
X	Blood, Volume 74, No.5, issued October 1989, J. Lotem et al., "Regulation of megakaryocyte development by interleukin-6", pages 1545-1551 see entire document.	3,4,7,8 10,12,13 15,17,22- 26

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
X	Blood, Volume 73, No.7, issued 15 May 1989, D. Rennick et al., "Interleukin-6 interacts with interleukin-4 and other hematopoietic growth factors to selectively enhance the growth of megakaryocytic, erythroid, myeloid, and multipotential progenitor cells", pages 1828-1835, see entire document.	3,4,7,8 10,12,13 15,17,22-26
X	Blood, Volume 73, No.6, issued 01 May 1989, E.J. Clutterbuck et al.,"Human interleukin-5 (IL-5) regulates the production of eosinophils in human bone marrow cultures; Comparison and interaction with IL-1, IL-3,IL-6, and GMCSF", pages 1504-1512, see entire document.	1-26

ATTACHMENT A (CONTINUATION OF PART VI)

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

The claims present the following independent and distinct inventions where I is the first group and will be searched in the event that no other fees are paid. Note that a search of any other additional groups requires payment of additional fees.

I. Compositions comprising or administration of G-CSF and IL-3, classified in class 424, subclasses 85.1 and 85.2 (claims 1, 2, 9, 10, 13, 14, & 18-23);

II. Compositions comprising or administration of G-CSF and GM-CSF, classified in class 424, subclass 85.1 (claims 1, 2, 9, 10, 13, 14, & 18-23);

III. Compositions comprising or administration of IL-5 and GM-CSF, classified in class 424, subclasses 85.1 and 85.2 (claims 5, 6, 11, 13, 15, & 16);

IV. Compositions comprising or administration of IL-6 with or without IL-3, classified in class 424, subclasses 85.1 and 85.2 (claims 3, 7, 8, 12, 13, 17, & 24-26);

V. Compositions comprising or administration of IL-6 with or without G-CSF, classified in class 424, subclasses 85.1 and 85.2 (claims 3, 4, 7, 8, 12, 13, 17, & 24-26);

VI. Compositions comprising or administration of IL-6 with or without GM-CSF, classified in class 424, subclasses 85.1 and 85.2 (claims 3, 7, 8, 12, 13, 17, & 24-26).

These six groups are directed to different inventions which are not so linked as to form a single general inventive concept. In particular, the use of the different combinations of lymphokines listed above would be expected to stimulate hematopoiesis by different mechanisms giving different end results and the search for any one group would not result in a complete and thorough search for any other group.

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